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PROGRESS REPORT NUMBER I

DAPHNIA SURVIVAL STUDIES

NASA Research Grant No. NsG-519 to the University of Washington

For period beginning November 1, 1963, and ending May 1, 1964.

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RC #1

SUMMARY

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Experiments have been performed to define the most suitable conditions for a 7-day flight of Daphnia. The best culture medium developed is Chlamydomonas reinhardti culture diluted with aquarium water to 2.5×10^6 cells/ml plus calcium pantothenate (300 mg/l). Survival of 75% of the original animals and birth of approximately 24 young per female can be expected if other factors do not become limiting.

The optimum method of O_2 storage has not yet been determined. Both O_2 saturated medium and a gas storage chamber show promise. The CO_2 concentration appears to become toxic even before the O_2 becomes limiting. Use of more alkaline medium may eliminate this problem.

The flight chambers have been designed on a modular basis. Each module contains two chambers, each, for four pregnant Daphnia, plus their algal food, one chamber for algae only, and necessary preservation equipment. A modified module without preservation facilities will be used for the recovery of live cultures. Two preservation and one non-preservation module will require approximately 0.09 cu. ft. and will weigh less than 4 lbs.

Morphological studies have been concerned with (a) normal morphology and embryology (Plate I), (b) feasibility of the use of exoskeleton markings as an index of cellular change (Plate II), and (c) induction of cyclomorphosis. Mapping of exoskeleton markings seems to be a promising method of determining changes in cell size and shape while the animals are unobserved in space. Pictures of normal morphology, embryology, and exoskeleton structure are shown.

A collection of ten species has been maintained to provide alternate species with a variety of environmental requirements.

Author

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INTRODUCTION

Research was started on November 1, 1963, rather than on October 1, the original date of the grant, since the NASA transmittal letter did not arrive until October 24, 1963, and the University of Washington did not grant provisional acceptance until November 1. Research has proceeded toward the experimental verification of the parameters of the experiment as proposed, toward the design of a suitable space flight module, and toward improvement of the reliability of the experiment.

Preliminary work was commenced immediately with on-hand facilities. Gathering of suitable personnel (see expenditure sheet) has gone more slowly due to the delay in the starting of the grant. The selection of specialized equipment has also gone slowly since there exist few instruments designed particularly for these problems. In almost every case equipment must be modified from the standard units.

In consultation with Ames personnel (Dr. W. Jones and Dr. J. Tremor) the data collection system was changed to automatic preservation of replicate samples rather than inflight monitoring as had originally been proposed. These changes, together with the assurance that the samples would be recovered, have permitted a change of emphasis from a simple survival and feeding experiment to a detailed physiological study.

RESEARCH PERFORMED

To date six experiments have been performed to measure the survival of animals in sealed containers to define the most suitable conditions for a 7-day flight. Studies have also been begun on the factors most likely to be altered by space flight: reproduction rates, embryological development, gross morphology, and cell size and distribution. The effects of O_2 and CO_2 concentrations which will develop under closed container conditions are being studied in order to assure that these secondary effects do not interfere with the prime objectives.

Flight Chamber

The flight chamber has been designed on a modular basis so that the total number of preserved samples can be conveniently altered, dependent on flight duration and frequency of sampling. (Fig. 1.) The module includes (a) two replicate chambers for animals with food (algae), each 50 ml, (b) one control chamber for algae only, 50 ml, (c) preservative storage chambers, 15 ml, (d) overflow chamber which may also serve for a gas reservoir prior to preservation, 15 ml, (e) timer to activate introduction of preservative, and (f) timer to time the application of the activation current. The module characteristics are shown in Table 1. A modified module for live recovery will not require the preservative chambers or the timers. If two perservation and one non-preservation modules are used, the over-all requirements will be approximately 0.09 cubic feet and less than 4 lbs. The only inflight monitorings anticipated are temperature, radiation and gravity measurements which are presumed to be part of the over-all system and are not included in this design.

Figure 1

DAPHNIA SATELLITE MODULE

Full Scale

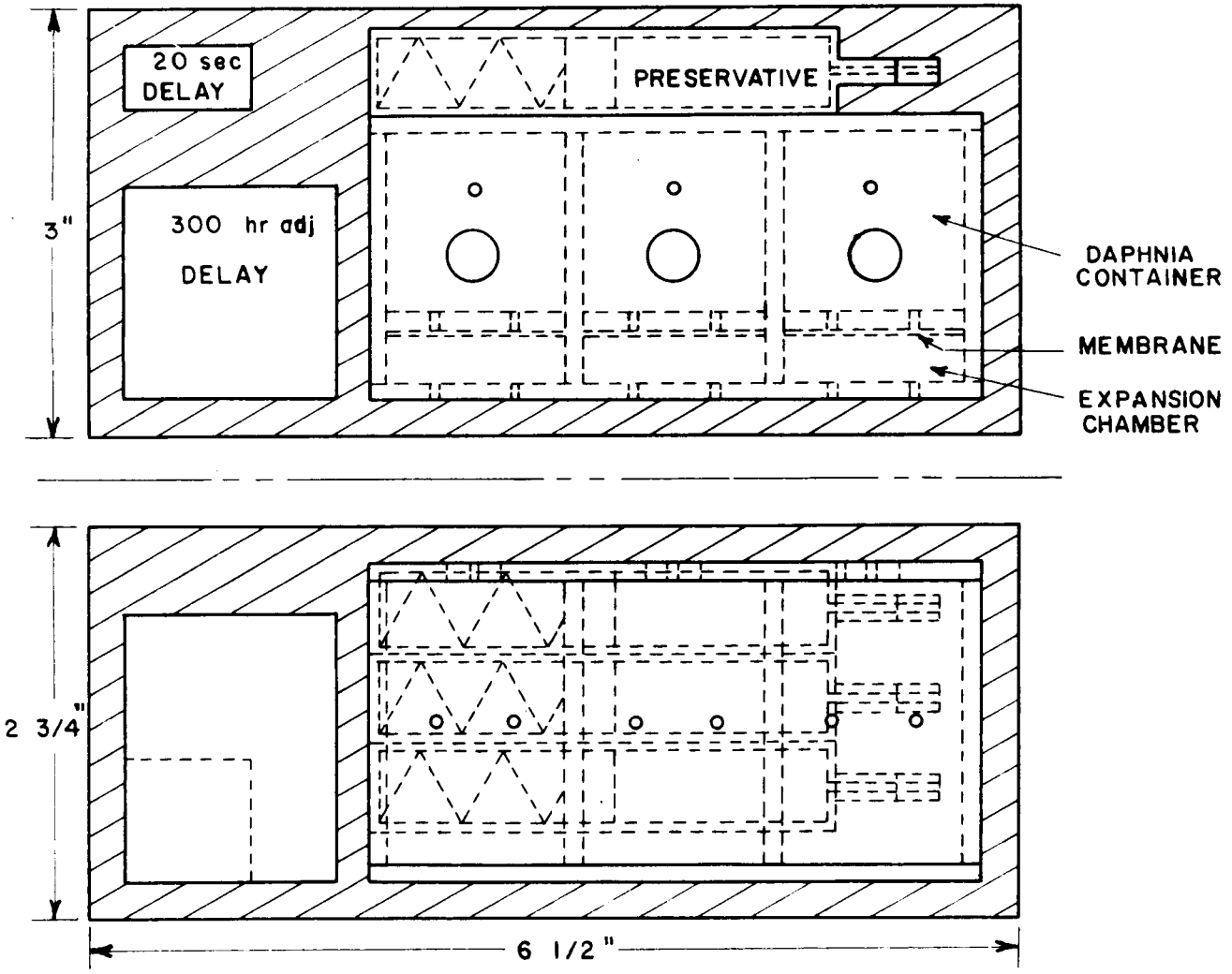

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Table 1. Flight Chamber for Aquatic Specimens. *Daphnia* Experiment.

Specifications for satellite module:	
Operating voltage:	24 v dc
Operating current:	60 ma continuous
	500 ma for one 20-second interval
Weight:	1.3 lbs.
Center of gravity:	No more than 1 inch from geometrical center.
Dimensions:	Parallelopiped 2-3/4" x 3" x 6-1/2"; 0.031 cu. ft.

Subject to later modifications, a mock-up of the module may be constructed and tested.

Survival and Reproduction under Simulated Experimental Conditions

Survival of 74 groups of four pregnant females, each group housed in individual containers, were measured under a variety of conditions for a duration of seven days. The general flight conditions are tentatively defined as: volume, 62 ml, either completely filled with liquid or with various proportions of liquid and gas phases; temperature, 20°C, and darkness. Adequate food, sufficiently high concentration of O_2 and sufficiently low CO_2 levels were identified as the paramount factors controlling survival and reproduction. Satisfactory culture conditions can now be defined but further studies are recommended to increase the reliability.

Food and Culture Media

Tests to date indicate the best medium to be *Chlamydomonas reinhardti* culture diluted with aquarium water to 2.5×10^6 cells/ml plus calcium pantothenate (300 mg/l). Using a similar medium with only 1×10^6 cells/ml, 96% of the *Daphnia* survived to day 5, and 62% survived to day 7. Survival of 75% of the original animals can probably be assured, assuming that factors other than food do not become limiting.

Daphnia pulex remove suspended food (algae) from their culture medium by continuous filtration. The food intake is a function of the volume filtered and the cell concentration of the algae. In turn, the volume filtered is a function of the animal's size. An average young adult (1.4 mm) may be expected to filter 4 ml/day, while a newborn (1 mm) may filter 1.5 ml/day (Richman, 1958). The reduction in food concentration per day is a function of the volume filtered and the total volume. Therefore, in a 50 ml volume, four adult females would remove approximately 4 x 4 ml/50 ml = 30% of the available cells on day 1; 30% of the remaining cells on day 2, etc. This relationship more accurately described by the exponential decay rule $N = N_0 e^{-\lambda t}$ where λ is a function of the volume filtered compared to the total volume. The volume filtered increases with time due to growth of the original individuals and to the birth of young. Some egested cells become available for feeding a second time. If the food concentration is sufficiently high at the beginning, e.g., 2.5×10^6 cells/ml, there should be adequate food remaining at the end of seven days, $.2 \times 10^6$ cells/ml, Since the food organisms have an oxygen requirement of their own, excessive concentrations are undesirable.

Alternative methods of conserving food are to use either a species which feeds intermittently (rather than continuously) or a smaller species (see Culture Collection, discussed below). Larger containers or fewer animals are a less desirable means since this would reduce the amount of information to be gained relative to the experiment's weight. Continuous injection of food would probably be an unnecessary complication.

Chlamydomonas reinhardti, a unicellular motile alga, appears to be the best food organism. Chlorella pyrenoidosa, thermophilic strain #71105, was

originally proposed since this alga has frequently been suggested for photosynthetic gas regeneration sub-systems for sealed life support systems. Therefore, information on its mutation rate under orbital flight conditions would be extremely valuable. The Russians plan a similar study (Bolgarov, 1961). A mixture of these two algae could be used. The algal growth medium must be nontoxic to Daphnia (Taub and Dollar, 1964).

Reproduction is largely a function of the nutritional state of the Daphnia. Under the recommended conditions, the average brood size is about 8. Broods as large as 28 animals have occurred. At 20°C, broods are approximately 48 hours apart. Therefore, four pregnant females should produce 96 offspring during the seven-day flight. This number has been reached in experiments where the gas content of the liquid was in equilibrium with the air.

O_2, CO_2 and pH

Experimental Results: Two preliminary experiments were undertaken to test survival in sealed containers with and without a gas space, and with either air saturated or oxygen saturated media. At four days 82% survival in sealed containers with either 12 or 37 ml air spaces and 100% survival in unsealed containers was found (n = 16). Daphnia were able to tolerate O_2 (760 mm) saturated medium better than medium saturated with airoor the same pressure. Up to three days the presence of a gas space (12 ml) was necessary for the air saturated samples, but not for the O_2 saturated samples. Beyond the third and fourth days these experiments were markedly influenced by lack of food (the starting concentrations of the food were 0.16 and 0.6×10^6 Chlamydomonas cells/ml).

The results of a more complete experiment are shown in Table 2. The high CO_2 concentration may have become limiting even before the low O_2 concentration did. This CO_2 effect could probably be lessened by a more alkaline medium. Reproduction was reduced sharply in the sealed containers. It should be noted that the disappearance of O_2 and other changes were almost as great in the sealed containers without the Daphnia as those with animals. This uptake is probably due largely to bacteria and other extraneous contamination and due to Chlamydomonas only to a lesser degree. This indicates that O_2 conservation could be effected by eliminating the major portion of contamination. It is not feasible at this time to attempt bacteria-free cultures. The initial food concentration for this experiment was 1.2×10^6 cells/ml.

The O_2 uptake by an adult Daphnia averages 2.4×10^{-6} liters/day (Richman, 1958). Assuming an average of 50 animals x 7 days, less than 1 ml of O_2 would be required. In actual practice, however, the rate of O_2 uptake is much higher due to the uptake by the Chlamydomonas and by extraneous bacteria, etc.

O_2 storage can be accomplished in either the gaseous or dissolved state. Gas can contain approximately 33 times the O_2 content as an equal volume of water. It is recommended that if two phases are used, that they be separated by a gas permeable membrane since the separation of a gas-liquid system would be unpredictable under null gravity, and the animals might become trapped on air-water interfaces. To avoid this complication, a completely liquid system would be preferable. However, if an expansion chamber is required for the preservation system, it could simultaneously

Table 2

Results of O_2, CO_2, pH Experiment: Preservation of Replicate Samples

	Partial pressure* of dissolved gases										
	O_2			Free CO_2			pH			Animals***	
Day	Sealed with Daphnia	Sealed w/o Daphnia	Unsealed with Daphnia	Sealed with Daphnia	Sealed w/o Daphnia	Unsealed with Daphnia	Sealed with Daphnia	Sealed w/o Daphnia	Unsealed with Daphnia	Sealed with Daphnia	Unsealed with Daphnia
0	710	740	710	< 10	<9	<10	7.62	7.60	7.62	4 4	4 4
2	310 350	370	175 180	10 < 9	< 9	<9 < 9	6.83 6.82	6.84	7.05 6.88	13 12	20 19
4**	105 25	85	100 92	11 22	20	< 9 <9	6.30 6.40	6.99	7.03 6.85	11 20	35 21
6	50 60	45	125 145	19 19.5	19	<9 <9	6.60 6.55	6.51	7.36 7.49	23 29	32 44
7	~32 ~32	33	150 155	19 22	20	<9 -	6.50 6.46	6.44	7.31 7.46	16 19	96 109

Dead

* Measured at 37°C; at the experiment's temperature, 20°C, they would have measured somewhat lower.

** These samples were refrigerated for one day prior to O_2, CO_2, and pH measurements.

*** This represents the animals counted in samples preserved on the appropriate day. Except where indicated, the great majority of the animals were alive at the time of preservation.

serve as a gas storage area. An alternate means of increasing the O_2 content of the container is to saturate the medium with O_2 (760 mm) rather than air (160 mm O_2).

The production of CO_2 may be taken as approximately equal to the O_2 uptake. It will be distributed between free (dissolved) CO_2, bicarbonate and carbonate, the relative proportions depending upon the pH of the solution. Free CO_2 is probably the most toxic form. Above pH 8.0 practically no free CO_2 exists.

The acidity of the medium acts largely by influencing other reactions, especially CO_2 form and transport. Results of two experiments showed shifts in sealed containers from pH 7.15 to 6.8 and from 7.62 to 6.5 during seven days. (When continuous pH measurements are to be made, the medium must have a sufficiently high Na^+ concentration to avoid K^+ toxicity from leakage from the KCl solution in the electrode. Without this precaution a Leeds and Northrup micro pH electrode killed the animals in two days. Survival through seven days has been accomplished with redesigned media.)

Instrumentation: Since undesirable O_2 and CO_2 concentrations are the most likely source of interference in sealed experiments, the necessity of measuring and controlling these factors has been apparent from the beginning. Most standard chemical methods require excessively large samples and do not lend themselves to repetitive or automatic measuring. Instruments designed to measure these parameters by membrane electrodes on small liquid (blood) samples have recently been offered by several companies. These instruments are still in their early stages of development and in-lab modifications will be required. This equipment will be used to measure experimental conditions and to monitor ground controls, but will not be used in flight.

Equipment manufactured by the Radiometer Company was selected over three other competing systems. Its specifications are:

pH Channel	Temperature compensation	0 - 100°C
	Ranges	0 - 12 pH (divisions .1 pH)
		6.8 - 8.2 pH (divisions .01 pH)
pCO_2 Channel	Temperature compensation	0 - 100°C
	Basic range	10 - 190 mm Hg pCO_2
pO_2 Channel	Span control	3 x 10^{-8} to 10^{-12} A/mm Hg pO_2 in 10 steps and continuously
	Ranges	0 - 120 mm Hg pO_2 (divisions 1 mm)
		80 - 220 mm Hg pO_2 (divisions 1 mm)
		0 - 1200 mm Hg pO_2 (divisions 10 mm)
	Polarizing voltage	700 $\pm$ 50 mv

The other systems considered were Beckman S.P.I.D. Blood Gas Analyzer, the Beckman Spinco Model 160 Physiological Gas Analyzer and the Instrumentation Laboratory's IL-113-5. Our conditions of low temperature and small sample size demanded an instrument with greater flexibility than these instruments would allow. Equipment availability and reliability also were considered.

A Barber Coleman #23-C Gas Chromatograph has been made available on an option-to-buy basis. This shows promise for measurement of gas samples, and in-lab modifications for measuring dissolved gases in liquid samples are under way.

While waiting for the above equipment to arrive and be made operational, gas measurements have been made by the Pulmonary Function Laboratory of the University of Washington Medical School on a payment basis. This arrangement is not entirely satisfactory since their instrument is frequently not available and cannot be modified as required to measure sufficiently low CO_2 levels, smaller samples and at required temperature.

Morphological Studies

Morphological studies have been concerned with (a) normal morphology and embryology (Plate I), (b) feasibility of the use of exoskeleton markings as an index of cellular change (Plate II), and (c) induction of cyclomorphosis.

To assess the variation shown by normal animals and to develop the techniques required for studying animals recovered from space, photographic and permanent mounting techniques are being developed. The times of preservation will be determined by the rate of embryological development. The animals shown in Plate I, top, are normal except for the low number of embryos in the brood pouch. The complexity of such small organisms is demonstrated.

The embryos shown in greater magnification, bottom, were removed from the brood pouch for more detailed study. The organization of the complex multicellular embryo from the original single cell should provide the most critical test of intercellular coordination under space flight conditions. In particular, the lack of normal convection currents as a result of null gravity may be expected to interfere with the distribution of "organizer" substances which are presumed to coordinate the development of various portions of the embryo.

The shedding of the exoskeleton approximately every two days is a major event in the growth sequence. It remains suspended in the medium several days before being decomposed by bacteria. The exoskeleton is very noticeably cross-hatched with almost square markings on the lower head and carapace and long rectangular figures on the upper portion of the head, Plate II. It has usually been accepted that these markings conform closely

to the boundaries of the hypodermis cells which secrete the chiten exoskeleton (Anderson, 1933). If this is the case, the markings can be used as a means of mapping the changes which have occurred in cell size and shape while the animals were unobserved in space. This appears to be a promising method of measuring cell death or reproduction or change in size and form due to either radiation or null gravity. We are in the process of confirming the correlation between hypodermis cells and exoskeleton.

Cyclomorphosis is a phenomenon shown to a relatively high degree in certain species of Daphnia. It manifests itself as a change in head shape which is believed to respond primarily to turbulence of the water and, secondarily, to temperature (during the embryonic stages), and possibly to food (Brooks, 1947). We have had limited success in inducing head shape changes in Daphnia galeata mendotae by agitation of the medium. If this characteristic can be induced by excess gravitational forces, we may expect it to be altered by the absence of gravity. Since a European species, Daphnia cucullata is capable of undergoing far greater changes in head size and shape, we are attempting to procure live stocks.

Culture Collection

To provide species of a variety of sizes, oxygen requirements, temperature tolerances and feeding habits, a type culture collection has been supported in part by this grant. Expenses of collection, etc., have been supported by other sources. The collection includes 26 strains of the following ten species: Ceriodaphnia reticulata, Chydorus sphaericus, Daphnia ambigua, Daphnia galeata mendotae, Daphnia magna, Daphnia pulex, Daphnia schødleri, Pleuroxus denticulatus, Scapholeberis kingi and Simocephalus serrulatus. These range in size from Daphnia magna of 3.5 mm

to Chydorus sphaericus of only 0.5 mm. Daphnia magna is extremely hardy, but inconveniently large. Daphnia pulex, the species originally proposed, is smaller, 2.4 mm, has been intensively studied, and has been the subject of numerous other studies in our laboratory. It lacks the ability to undergo cyclomorphosis (discussed above) which may respond to changes in gravity. Daphnia galeata mendotae undergoes cyclomorphosis to a limited extent.

References

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Richman, Sumner. 1958. The transformation of energy by Daphnia pulex. Ecological Monographs, 28:273-291.

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REPORTS AND PAPERS

James J. Orsi, Frieda B. Taub and Alexander M. Dollar, "Cladocera Culture Collection," will be presented by Mr. Orsi at the annual meeting of the American Society of Limnology and Oceanography, June 23-25, 1964, at Vancouver, B. C. (Canada). (This research has been supported in part by this grant and in part by a Public Health Service Grant.)

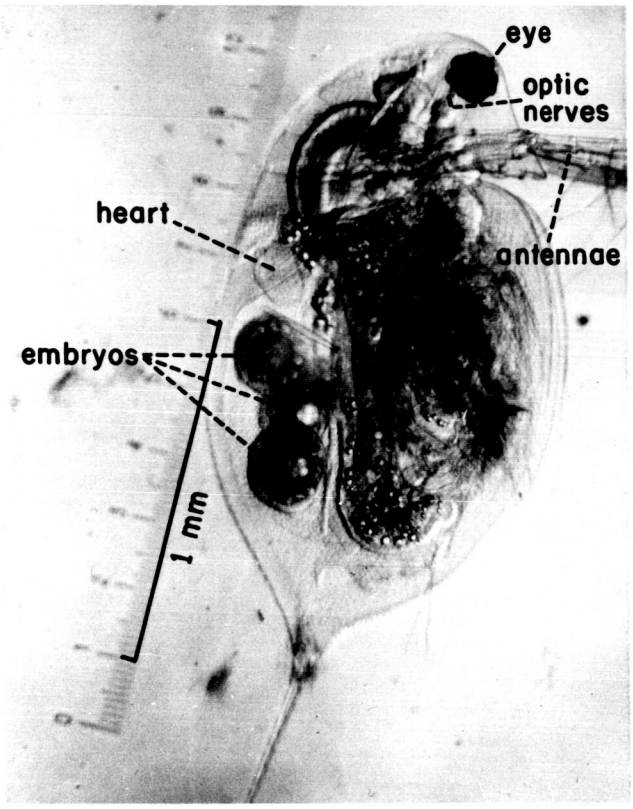
It is anticipated that two papers will be published in the biological journals and possibly one report will be made in a NASA publication.

A preliminary report and a collection of germaine reprints were sent to Ames Research Center on November 20, 1963.

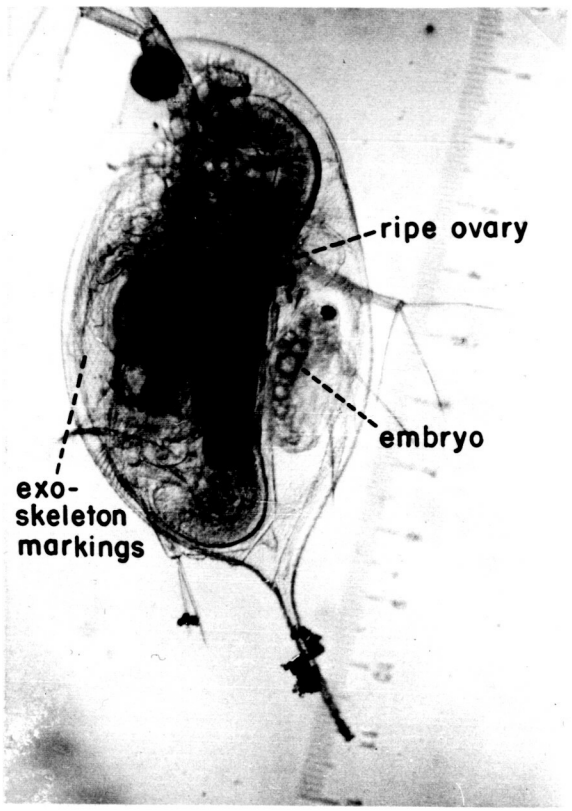
DIRECTION OF FUTURE RESEARCH

Research will proceed along the present lines. Most of the remaining necessary equipment will be procured during the next two months. During the remaining time, increased time will be spent by the principal investigator and at least one part-time employee may be replaced by a full-time employee. The terminal date is interpreted as on or about November 1, 1964, one year from the effective starting date. Present funding will be adequate through the second six months of the grant.

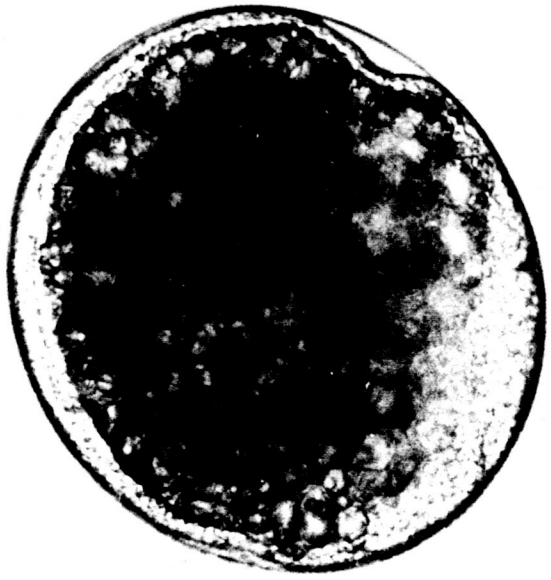
The goals of the project are to develop methods which will provide maximum reliability and to gather sufficient background to assure maximum information from the experiment. A necessary prerequisite of the primary goals is the development of instrumentation to measure O_2, CO_2 and pH conditions in the sealed aquatic test chambers.



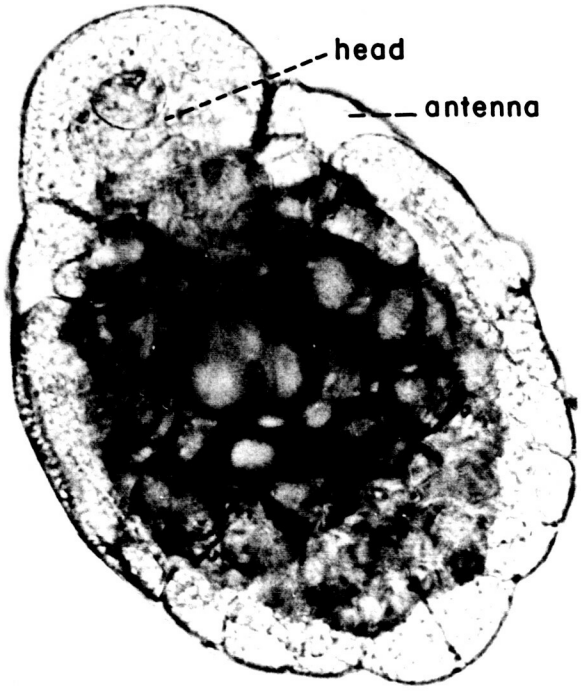
Female with early embryos
50 X



Female with late embryo, ca. 42 hrs.
50 X

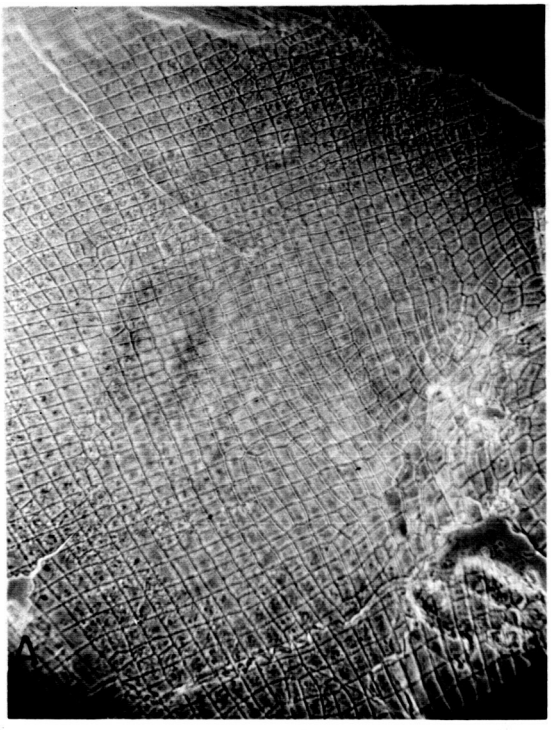


Early embryo , 13 hours 160X

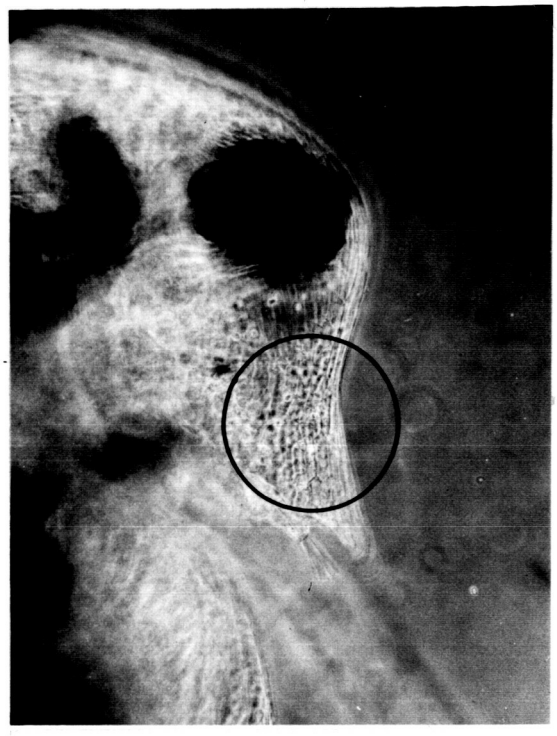


Intermediate embryo, 30 hours 160X

Plate I, Daphnia pulex, Morphology and Embryology

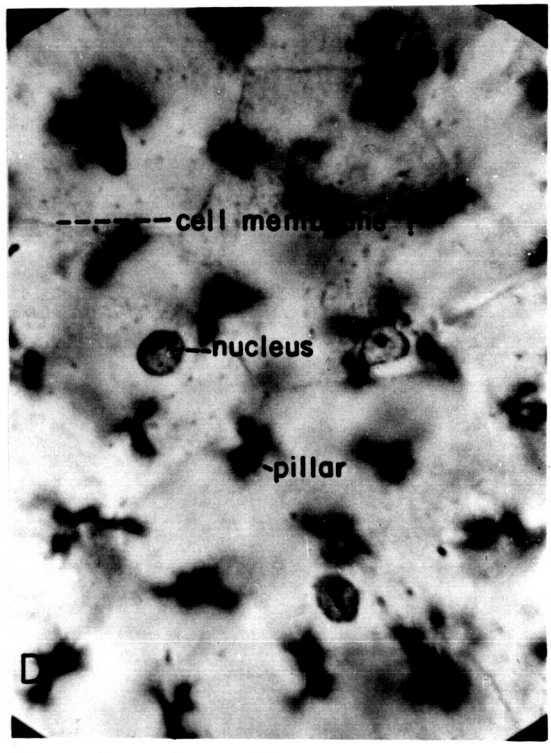


Exoskeleton markings on carapace
(Live material) 500X



Exoskeleton markings on head
(Live material) 100X

Exoskeleton markings and
hypodermis cells (secreting cells)
(Live material) 2000X



Hypodermis cells
(Sectioned, H and E stained) 4000X

Plate II, Daphnia pulex, Correspondence of Exoskeleton Markings and Cellular Structure